AMERICAN JOURNAL OF PHYSICAL ANTHROPOLOGY 104:1–21 (1997)

Hormone Measures in Finger-Prick Blood Spot Samples: New Field Methods for Reproductive Endocrinology

CAROL M. WORTHMAN* AND JOY F. STALLINGS
Laboratory for Comparative Human Biology, Department of Anthropology, Emory University, Atlanta, Georgia 30322

KEY WORDS FSH; LH; PRL; testosterone; estradiol; DHEAS; androstenedione; SHBG; puberty; cortisol; saliva; human behavior

ABSTRACT Comparative endocrine studies have notably advanced understanding of ecological factors that contribute to variation in human reproductive function. Such research has relied on methodological advances that permit hormone determinations in samples that are easily and safely collected, stored, and transported, most recently on measurement of steroids in saliva. This report seeks to further expand the scope of endocrine research by demonstrating the value of blood spot samples collected by finger prick. As a sampling strategy, finger-prick blood spot collection offers the advantages of short collection time, low invasiveness, repeatability, absence of postcollection processing, low biohazard risk, and ease of sample storage and transport. We document good sample stability and present sensitive assay methods for a range of steroids and proteins (FSH, LH, PRL, T, E2, DHEAS, androstenedione, cortisol, SHGB) in blood spots that require sample volumes of 3–12 µl and display good reliability, specificity, precision, accuracy, and convertibility of results to plasma/serum equivalent concentrations. Laboratory evaluation was augmented by a feasibility study at a remote site in Papua New Guinea that confirmed validity and stability of blood spot collections under field conditions. Research applications of blood spot sampling are illustrated with a series of studies, including cross-sectional surveys for developmental and life span endocrinology, a longitudinal, population-based developmental epidemiologic study of puberty, and serial sampling in a dynamic study of neuroendocrine response to suckling. We conclude that the sampling features and wide range of measurable biomolecules of blood spots do constitute a methodological advance for endocrine research. Am J Phys Anthropol 104:1–21, 1997.

During the past two decades, measurements of salivary steroids have enhanced endocrine research (Malamud and Tabak, 1993), broadening the scope of study to previously inaccessible populations and study sites, such as remote field locations, homes and work places, exercise facilities, and educational settings. The advantages of salivary assessments of hormones over blood stem primarily from the ease of sample collection, providing a completely painless sampling technique that is minimally disruptive to the daily routine and highly acceptable to most subjects. Steroid hormones are, moreover, quite stable in unchilled, preserved saliva specimens, which eases storage constraints.

Despite these manifest advantages, salivary measures have certain drawbacks. In

Contract grant sponsor: W.T. Grant Foundation; contract grant number 92-1489-92, 94-1489-2; Contract grant sponsor: University Research Committee of Emory University; Contract grant sponsor: NIMH; contract grant number MH48085.

*Correspondence to: Carol M. Worthman, Department of Anthropology, Emory University, Atlanta, GA 30322.

Received 12 September 1996; accepted 11 June 1997.

fact, salivary samples present several logistical and physiological limitations. Sample vials are bulky, breakable, potentially leaky, and unwieldy to transport; such considerations are most significant for field workers at remote sites who must transport saliva vials long distances in a range of environmental circumstances (terrain, climate, altitude) using diverse means of conveyance (e.g., foot, air, or llama). More important scientifically is the fact that saliva does not reflect circulating concentrations of peptide or protein hormones, binding proteins, conjugated steroids, or other membrane-insoluble constituents. Saliva represents the free steroid fractions rather than that bound to carrying proteins and thus is thought to represent the bioavailable moiety. While this has been viewed as an advantage, salivary studies provide limited estimates of total output by peripheral glands (gonads, adrenals) and preclude evaluation of central neuroendocrine regulation. Further, that merely 2–8% of circulating concentrations are present in saliva places severe technical demands on assays for steroids of low concentrations (e.g., estradiol) and prevents their use in developmental studies.

In 1990, faced with these limitations, we commenced development of new methods that would be similar to salivary collections in noninvasiveness and acceptability but that would obviate some of the problems and limitations we had encountered by relying solely on salivary methodology when blood samples from venipuncture were impossible. The ability to study central neuroendocrine regulation was crucial to our cross-population investigations of developmental endocrinology and factors influencing maturational timing and sociobehavioral and environmental effects on adult reproductive function and stress. Since most of our studies involved remote field settings, an important criterion for any new method was sample stability to allow some sample storage at the site since immediate transportation to the laboratory was rarely possible. Based on published methods for blood spot thyroid stimulating hormone (TSH) and their widespread use in neonatal screening (Brombacher et al., 1988; Torresani and Scherz, 1986; Waite et al., 1987), for blood spot prolactin (PRL) assays (Bassett et al., 1986), and for some blood spot steroids (Egan et al., 1989; Hofman et al., 1985; Kraiem et al., 1980; Petsos et al., 1986; Thomson et al., 1989), the mode of sampling we chose was blood spot by finger prick.

After 6 years of effort in method development, we now have blood spot radioimmunoassays (RIAs) and fluoroimmunometric assays (FIAs) for determination of gonadal (testosterone (T), estradiol [E2]) and adrenal (androstenedione (A), dehydroepiandrosterone-sulfate (DHEA-S), and cortisol [C]) steroids, pituitary hormones (follicle stimulating hormone (FSH), luteinizing hormone (LH), and PRL), and the primary sex hormone carrying protein (sex hormone binding globulin [SHBG]). These methods have met our sample collection and handling criteria as well as our scientific interests in achieving endocrine profiles of both steroid and protein hormones from a single collection in a field setting, interests heretofore met only by serum or plasma samples from venipuncture.

Although blood collection is inappropriate for some populations, these methods provide a means by which blood samples can be obtained where the major concerns have been invasiveness, storage, and handling. The purpose of this paper is to provide other researchers with methodological information and examples of studies performed in our laboratory that demonstrate their application. Our own experience suggests that these methods will prove useful not only for anthropologists but also for a wide range of epidemiological, clinical, psychobiological, and interdisciplinary research. They furthermore expand the range and power of endocrine investigation of normal individuals in everyday settings.

COMPARISON OF BLOOD SPOTS WITH SALIVA AND SERUM

Collections of finger-prick samples are minimally invasive since the devices used are designed for repeated collections by diabetics for glucose self-monitoring. The assays themselves require very little blood; in fact, all of the aforementioned analytes can be measured simultaneously from collection of four drops of blood (200 µl or four circles

about the size of a dime). Unlike saliva, sample volume for blood spots is low because hormone levels are generally one or two orders of magnitude higher in blood than in saliva. Furthermore, advances in assay systems yield ever more sensitive measures, particularly for proteins, requiring concomitantly diminished sample volumes. With highly sensitive RIAs and FIAs (Haavisto et al., 1990; Lovgren et al., 1985; Stenman et al., 1985), measurements can be performed on very small sample sizes. Although saliva collection is totally painless, up to 15 min or more of subject time is needed to collect the 5 ml volumes often required for analysis. Subject collection time for finger pricks, on the other hand, is more on the order of a couple of minutes.

One reccurring problem we and others have experienced with saliva collections is blood contamination, a highly prevalent problem in field settings where diet type and oral hygiene may conduce to bleeding gums (Campbell, 1994). Because of the large blood-saliva disparity in hormone concentrations, even minute amounts of blood contamination can cause highly inaccurate results. Blood in saliva can be detected by techniques such as dipsticks used to identify blood in urine (VWR Scientific Products, Stone Mountain, GA) so that contaminated samples can be discarded. This strategem is plagued by false positives (Beall et al., 1992) and can jeopardize the study by reducing the sample size and eliminating important, irreplacable samples. Others have reported a technique that may be useful in separating blood-source hormones from saliva (Campbell, 1994), but these techniques increase analytical time and cost.

Fewer factors compromise the validity of blood spot samples for analysis. Blood from the finger prick is dropped directly onto specially designed filter paper (#903 Schleicher & Schuell (S&S), Keene, NH) that absorbs the blood uniformly to make a homogeneous, evenly dispersed blood spot that is essential for accurate analysis (NCCLS, 1992). Sample collection errors that can generate unsuitable samples include 1) incomplete absorption, which occurs when an inadequate amount of blood is smeared on the paper surface (see details of blood collection that follow), 2) nonuniform samples, when one blood drop is applied on top of another, and 3) exposure of samples to direct sources of heat such as sunlight (or to blood-eating insects!).

Since release of many hormones is pulsatile (Dunkel et al., 1990; Marshall et al., 1991; Stenman et al., 1985; Wu et al., 1991), one limitation of serum/plasma samples has been that a single-point sample may be taken during the peak of the secretory pulse and thus fail to represent tonic or mean concentrations (Campbell, 1994; Ellison, 1988). The invasiveness of venipunctures or catheterization in field settings have left few alternatives to this problem. Finger pricks, on the other hand, allow multiple collections; we have used two collections 20 min apart in our epidemiologic studies of the associations between pubertal development and mental health among US adolescents (Angold et al., 1995) and three serial collections 20–25 min apart in our investigations of lactational infertility among nursing Nepali women (Stallings et al., 1996). Finger pricks also allow self-sampling; in our study of attitudinal and hormonal changes in expectant fathers (Worthman and Stallings 1994; Worthman et al., 1991), participants collected blood spot samples thrice weekly for 9 weeks, storing samples in their home refrigerator until weekly pickup by investigators.

Steroids in saliva reflect the portion of circulating hormone that is able to diffuse across acinar cells lining the salivary gland (Riad-Fahmy et al., 1987; Vining and McGinley, 1987). Since this process is analogous to the diffusion of steroids across cell membranes of target cells, a reported advantage of salivary over plasma hormone levels is that the former reflect the biologically active (the free or unbound) portion available to target cells. Indeed, several reports document strong correlations between salivary steroid concentrations and target tissue function (Beall et al., 1992; Ruutiainen et al., 1987; Osredkar et al., 1989).

Blood hormone assays reflect both bound and free forms, and several studies have yielded strong correlations between total plasma and salivary free steroid levels (Rilling et al., 1996; Sannikka et al., 1983; Wang

TABLE 1. Source of reagents, type of assay, range of blood spot standards, and duration of sample stability by temperature[1]

Hormone	Kit manufacturer	Assay	Range of standards	Sample stability		
				Room temperature	4°C	37°C
Pituitary hormones						
FSH[2]	Wallac, Inc.	FIA	0.5–128 IU/L	8 weeks	8 weeks	1 week
LH[3]	Wallac, Inc.	FIA	0.75–125 IU/L	8 weeks	8 weeks	1 week
PRL[4]	Wallac, Inc.	FIA	1.25–125 ng/mL	3 weeks	8 weeks	1 week
Gonadal hormones						
T	Binax (South Portland, ME)	RIA	6–1,000 ng/dL	3 weeks	8 weeks	5 days
E2	Pantex (Santa Monica, CA)	RIA	5–1,500 pg/mL	3 weeks	8 weeks	NS
Adrenal hormones						
DHEA-S	Pantex	RIA	25–4,000 ng/mL	4 weeks	8 weeks	1 week
A	DSL (Webster, TX)	RIA	0.05–5 ng/mL	4 weeks	8 weeks	2 weeks
C	Pantex	RIA	0.5–16 µg/dL	4 weeks	8 weeks	NS
Binding protein						
SHBG	Wallac, Inc.	FIA	3.125–100 nmol/L	2 weeks	8 weeks	NS

[1] A, androstenedione; C, cortisol; DHEA-S, dehydroepiandrosterone sulfate; E2, estradiol; FSH, follicle stimulating hormone; LH, luteinizing hormone; NS, not stable; PRL, prolactin; SHBG, sex hormone binding globulin; T, testosterone.
[2] Calibrated against the second IRP (78/549).
[3] Calibrated against the WHO second International Standard (80/552).
[4] Calibrated against the WHO third International Standard (84/500).

et al., 1981; reviewed in Malamud and Tabek, 1993). For example, in a recent study of 218 Zimbabwean male adolescents 11–23 years of age (Rilling et al., 1996), we found a high correlation between matched salivary and blood spot T levels (r = .83) and expected increases in both salivary and blood spot T across pubertal stage (assessed by self-reported Tanner genital stage ratings). Although blood spot T levels were slightly more effective than salivary levels in distinguishing the stage of genital development, the difference was not sufficient to establish the superiority of one measure over the other as an index of the bioavailability of circulating plasma T.

SHBG is the primary carrying protein of both T and E2 and the most significant determinant of the proportion of free steroid in circulation (Anderson, 1974; Dunn et al., 1981; Pardridge, 1981; Petra, 1991; Siiteri et al., 1982). Certain physiological states (e.g., pregnancy, obesity, puberty, and increased energy expenditure) and pathological conditions (e.g., cirrhosis of the liver and Cushing's disease) alter SHBG and thus the ratio of free vs. bound steroids (Anderson, 1974; Blank et al., 1978; Carter et al., 1983). Under such conditions, blood spot measures of both SHBG and sex steroid are useful for determining a "free index," an indicator of the biologically available steroid portion, by calculating a ratio between the total steroid level and SHBG. Such measures also allow investigation of whether individual and population variation in SHBG may contribute to variation in endocrine function and regulation.

One important advantage of saliva samples over serum and plasma has been stability under field conditions. Ellison (1988) determined that steroids in saliva treated with 0.01% sodium azide and left at room temperature for as long as 6 months showed no significant change in concentrations. By contrast, serum and plasma samples must be frozen within a 24 h period to insure stability for most analytes. Therefore, for field collections, the substantial time, labor, and equipment involved in blood sample processing, storage, and transport (on dry ice or in a cryogenic freezer, assuming sources of processed gasses are available near the site) deter more frequent use of blood samples.

The hormones we have measured in blood spots, although not stable as long as saliva at room temperature, remain stable for up to 3 weeks as long as they are kept dry and out of direct sunlight. In humid environments, storage with dessicant prevents mildew of the samples. Refrigerated samples (4°C) are stable for several weeks (see Table 1 for hormone-specific stability results) and can be removed for transport at room temperature without degradation. Freezer-stored

samples are stable for at least a year. Since samples are dry, the possibility of spills is eliminated, and papers can simply be carried in zip-lock bags, making transport of blood spot samples considerably easier than transporting saliva or serum. Moreover, drying of samples destroys HIV and hepatitis viruses and thus substantially reduces biohazard (Knudsen et al., 1993; Meredith and Hannon, 1993).

A final advantage of serum measures over saliva is the enormous literature available based on serum/plasma assays. Blood spot values are highly correlated with plasma values and thus can be converted to plasma equivalents using linear regression coefficients (Table 4), providing researchers with the advantage of comparability to this rich endocrine literature.

METHODOLOGY

Sample collection

The materials needed for collection of finger pricks are minimal. Samples are collected on filter papers (#903; S&S) highly standardized to absorb blood in a homogeneous manner so that uniform punches from any section of the sample will yield the same quantity of blood (NCCLS, 1992). After the fingertip is wiped with an alcohol swab, the finger is pricked with a lancet (Unilet Blood Lancets; VWR Scientific Products, Stone Mountain, GA) fitted into a spring-type auto lancet device (Autolet Lancet Mark II; VWR Scientific Products) designed to minimize pain to the subject. In fact, this device is used by diabetics for chronic self-sampling for blood glucose monitoring. Where hands are calloused or where investigators have little experience in finger-prick collection, very slightly larger lancets [Microtainer Safety Flow Lancet; VWR Scientific Products] should be used. Whichever device is employed, it must be pressed firmly to the finger for adequate penetration. When blood flow begins, the first bit is wiped away with a tissue since this drop may contain tissue fluids. The next drops are placed on each of five preprinted circles on the S&S paper that has been previously labeled with appropriately identifying subject information. The sample is then left at room temperature to air-dry, usually 3–4 h, depending on humidity levels. After drying, the card is placed in a zip-lock bag and stored at room (22°C) or refrigerator temperature (4°C) for the times specified in Table 1 or frozen (−20°C) until shipment to the laboratory for analysis.

Moderately experienced investigators can reliably obtain five drops per puncture. The greatest potential source of error in sample collection comes from application of the sample to the paper. The paper is designed to wick away the blood drop without the finger actually touching the paper. In other words, the finger is held slightly above the paper and the blood is simply dropped onto the printed circle, penetrating both sides of the paper. In cold climates, care should be taken not to allow the samples to freeze and thaw, while in warm climates samples should be kept away from direct sunlight and in a relatively cool area (i.e., by placing them in the shade). During rainy seasons or highly humid conditions, dessicant should be used to avoid mildew. Papers should not be compressed before use, because compression may affect the papers' absorbent properties. A word of caution concerning insects: one researcher had the unfortunate experience of having exposed samples eaten by flies while air-drying, so placing them in a cardboard box while drying may be prudent.

Blood spot hormone assays

The following section is provided primarily for readers familiar with RIAs and FIAs who are interested in incorporating the methods in their own laboratories or who plan to collaborate with other laboratory scientists.

Preparation of blood spot controls and standards. Commercial control sera are purchased from Bio-Rad, ECS Division (Anaheim, CA), and standards are supplied by the kit manufacturers. Controls are prepared according to the manufacturer's instructions by adding 5 ml deionized water to the lyophilized product, allowing it to stand at least 15 min to reconstitute, and then inverting it several times to thoroughly mix the contents. Standards are supplied in a serum-based liquid form so reconstitution is not necessary.

TABLE 2. Number of blood spot punches and volume of buffer used in elution of blood spots and dilutions of kit reagents[1]

Assay	Elution of Standards, Controls, Samples		Dilution of antibody[2]	Incubation time (min) with tracer	Dilution of second antibody
	Number of punches per well	Assay buffer volume (µL)			
Fluoroimmunometric assays					
FSH	1	200	(1:100)	30	
LH	1	200	(1:150)	15	
PRL	1	200	(1:75)	90	
SHBG	1	500	(1:50)	120	
Radioimmunoassays					
T	4	300	(1:4)		(1:2)
E2	4	200	(1:8)		(1:4)
DHEA-S	1	75	ND		ND
A	4	200	(1:6)		(1:2)
C	1	100	(1:4)		(1:4)

[1] ND, no dilution.
[2] FIA: europium-antibody solution; RIA: first antibody

Blood spot standards and controls are prepared by mixing reconstituted controls and standards 1:2 with red blood cells that have been washed three times with normal saline. For some assays, whole blood pools from EDTA-anticoagulated venipuncture collections are also used as controls. Five aliquots (50 µl each) of prepared blood standards and controls are dropped onto the sample collection papers, allowed to dry at room temperature overnight, and then stored in an air-tight container at −20°C. The manufacturer provides expected values for the commercial controls and blood spot values for controls, and standards are half that expected for serum or plasma since they are diluted 1:2 with red blood cells. Blood spot controls and standards are stable for at least 1 year when stored frozen at −20°C.

Principles of the assays. The blood spot PRL, FSH, LH, and SHBG assays are modifications of commercially available FIA kits for measurement of these hormones in serum or plasma (DELFIA; Wallac, Inc., Gaithersburg, MD). The principle involves the direct determination of hormonal levels by using two monoclonal antibodies directed against separate hormonal antigenic sites, one antibody immobilized on microtitre wells and the other europium-labeled. Europium ions are dissociated from the antibody complex, which then fluoresces in conjunction with a chelating agent. Shaking and washing of assay strips and time-resolved fluorimmetry are performed by instruments purchased from the kit manufacturer (Wallac, Inc.).

The blood spot T, E2, A, DHEA-S, and C assays are modifications of commercially available serum/plasma RIA kits (see Table 1 for manufacturers). The basic principle is competition between a radioactive and nonradioactive antigen for a fixed number of antibody sites (Yalow and Berson, 1971). The concentration of hormone in the sample is inversely proportional to the amount of ^{125}I-labeled hormone bound to the antibody. The free and bound antigens are separated by the addition of a second antibody–polyethylene glycol (PEG) reagent followed by centrifugation. After the supernatant is decanted, the amount of radioactivity in the antibody bound tracer complex is measured in a gamma counter (Cobra Gamma Counter, model 5005; Packard Instrument, Co., Downers Grove, IL).

Preparation of reagents. For all FIAs, europium-labeled antibodies are diluted with buffer supplied in the kits. Dilutions of antibodies and separating reagents for the RIAs are made with Dulbecco's phosphate-buffered saline (Gibco, Grand Island, NY), pH 7.4, containing 0.1% gelatin. This working buffer is prepared by adding 100 mg of gelatin to 100 ml of Dulbecco's buffer and heating to 45°C to dissolve. Table 2 shows the dilutions made for the reagents in each assay. All other reagents required for each assay are supplied in the respective kit in ready-to-use form.

Elution of blood standards, controls, and samples

Fluoroimmunometric assays

FSH, LH, and PRL assays. Blood is eluted from the sample paper using kit assay buffer. The assay strips, consisting of 12 microtitre antibody-coated wells, are rinsed once with wash solution using an automatic washer (Platewash automatic washer, model 1296-024; Wallac, Inc.). Blood spot standards, controls, and samples are removed from the freezer, and duplicate 2.5 mm punches (using a hole punch available at local office supply stores), equivalent to 3 µl whole blood, are transferred to the wells (one punch per well) with tweezers. Within 30 min of placing the spots, 200 µl assay buffer is delivered to each well. With an automatic plateshaker (Plateshake automatic shaker, model MPS-4; Wallac, Inc), the plates (consisting of eight microtitre strips) are rotated at 150 rpm for 10 min at room temperature. The wells are then placed in an air tight container in the refrigerator (4°C) overnight.

SHBG assay. Blood spot standards, controls, and samples are removed from the freezer, and a 2.5 mm punch from each is transferred to a 12 × 75 mm glass tube. Kit assay buffer (500 µl) is added to the tubes; the tubes are shaken on the automatic shaker for 10 min at 50 rpm at room temperature, covered tightly with parafilm, and then incubated overnight at 4°C.

Radioimmunoassays. Blood is eluted from the sample paper using working buffer. Buffer volume and number of punches vary with each assay and are listed in Table 2. Whole blood sample volumes per well range from 3–12 µl. The specified number of 2.5 mm punches is transferred to labeled 12 × 75 mm glass tubes, and a volume of working buffer (see Table 2) is added to each tube. The tubes are covered with parafilm and rotated on the automatic shaker at 50 rpm for 1 h at room temperature. After shaking, the tubes are placed in a 4°C refrigerator overnight.

Assay procedures

Fluoroimmunometric assays

FSH, LH, and PRL assays. Following overnight incubation, the wells are removed from the refrigerator and placed on the automatic shaker at 50 rpm for 1 h. The dilution of europium-labeled tracer solution is prepared as specified in Table 2 using kit assay buffer. Following the 1 h shaking, the filter paper discs are removed from the wells using vacuum aspiration with a pasteur pipette. The strips are washed twice with kit wash buffer using the automatic washer, and 200 µl of tracer solution is added to the wells. The strips are then placed on the automatic shaker at 150 rpm for 2 min and then at 50 rpm for the time specified in Table 2. Each strip is washed six times using the automatic platewasher, 200 µl of kit enhancement solution is added to each well, and the strips are rotated on the automatic shaker at 50 rpm for 5 min. After shaking, the wells are incubated at room temperature for 10 min, and the fluorescence is measured in the fluorometer (Arcus time-resolved fluorometer, model 1230; Wallac, Inc.). Concentrations are interpolated from the standard curve using a linear/log data reduction method and converted to plasma equivalents derived from regression analysis of matched blood spot/plasma samples (see Table 4 for regression coefficients for each assay). All data analyses are performed using RIA-Smart and Expert QC Software (Packard Instrument Co., Downers Grove, IL).

SHBG assay. Following overnight incubation, the tubes are removed from the refrigerator and placed on the automatic shaker at 50 rpm for 1 h. The assay strips (12 wells per strip) are rinsed once with kit wash solution using the automatic washer. A 25 µl aliquot of each eluate is transferred to duplicate wells, and 100 µl of assay buffer is added. The strips are shaken on the automatic shaker at 50 rpm for 2 h and then washed twice with wash solution. The dilution of europium-labeled tracer solution is prepared as specified in Table 2, and 100 µl is added to each well. The strips are shaken for 2 h at 50 rpm and then washed six times

TABLE 3. Assay-specific volumes (μL) of blood spot eluates and reagents, incubation and centrifugation times (min) and temperatures (°C) for each radioimmunoassay[1]

Protocol step	Specification	T	E2	DHEA-S	A	C
Pipet	Blood spot eluate	100	75	50[2]	75	40
	Tracer	50	20	100	50	30
	First antibody	100	500	500	100	100
First antibody incubation	Time	ON	ON	30	40	30
	Temperature (°C)	RT	RT	RT	37	37
Pipet	Second antibody reagent	500	500	500	500	500
Second antibody incubation	Time	20	60	10	15	10
	Temperature	RT	RT	RT	RT	RT
Centrifugation	*g*	2,300	2,300	2,300	2,300	2,300
	Time	60	60	30	20	60
	Temperature	4	22	22	22	22

[1] ON, overnight (18–24 h); RT, room temperature (20–24°C).
[2] A further 1:10 dilution of the blood spot eluate is made with kit buffer (50 μL eluate plus 500 μL kit diluent); 50 μL of this dilution is used in the assay.

with wash solution using the automatic washer. Enhancement solution (200 μl) is added to each well, and the wells are shaken on the automatic shaker at 50 rpm for 5 min incubation. Following shaking, the wells are incubated at room temperature for 10 min, and the fluorescence is measured in the fluorometer. Concentrations are interpolated from the standard curve using a linear/log data reduction method and converted to serum equivalents derived from regression analysis of matched blood spot/serum samples (see Table 5 for regression coefficients). Note that EDTA-anticoagulated blood cannot be used in this SHBG assay; therefore, reported comparisons of SHBG measured in whole and processed blood are based on matched blood spot and serum samples.

Radioimmunoassays. Following overnight incubation, the tubes are removed from the refrigerator and shaken on the automatic shaker at 50 rpm for 1 h at room temperature. Specific dilutions of reagents, volumes, incubation temperatures, and times are given in Tables 2 and 3. Following the 1 h incubation, a volume of the blood spot eluate, ^{125}I-labeled hormone, and diluted first antibody are added to duplicate 12 × 75 mm polypropylene tubes. The tubes are incubated, and then the antibody-bound and free antigen are separated using a second antibody/PEG reagent and centrifugation (Sorvall RC3C Centrifuge; Dupont, Wilmington, DE). After the supernatant containing unbound antigen is decanted, the amount of radioactivity in the pellet (containing the bound portion) is measured in the gamma counter. Concentrations are interpolated from the standard curve using a spline data reduction method and converted to plasma equivalents derived from regression analysis of matched blood spot/plasma samples (see Table 4 for regression coefficients for each RIA). All data analyses are performed using RIA-Smart and Expert QC Software.

TABLE 4. Comparisons of matched blood spot and plasma/serum hormone measures: Pearson correlation coefficients (r), number of comparisons (n), r-square, and regression coefficients from simple linear regression analysis used to calculate plasma/serum equivalents[1]

Assay	n	r	r-square	Regression formula
FSH	39	0.980	0.960	Y = 0.42 + 2.2(X)
LH	39	0.977	0.956	Y = 0.07 + 1.9(X)
PRL	35	0.985	0.970	Y = 0.73 + 2.0(X)
SHBG	20	0.934	0.873	Y = −10.0 + 2.0(X)
T	56	0.979	0.958	Y = 0.07 + 1.4(X)
E2	21	0.984	0.968	Y = 14.7 + 1.6(X)
DHEA-S	89	0.990	0.980	Y = 8.17 + 2.2(X)
A	51	0.978	0.956	Y = 0.03 + 1.4(X)
C	21	0.926	0.857	Y = 1.99 + 2.0(X)

[1] X, blood spot hormone concentration; Y, plasma/serum hormone concentration.

Assay performance

Sensitivity. The sensitivity of the FIAs is defined as the dose required to enhance fluorescing two standard deviations (SD) above zero dose, and the sensitivity of the RIAs is defined as the quantity of unlabeled hormone required to inhibit binding of tracer by an amount equal to 2 SD below the mean binding observed in the absence of unlabeled hormone. Sensitivity doses calculated for each FIA and RIA are listed in Table 5.

TABLE 5. Assay performance characteristics

Fluoroimmunometric assays	FSH	LH	PRL	SHBG
Sensitivity	0.13IU/L	0.26IU/L	0.11ng/mL	0.2nmol/L
Average % recovery	89.7	100.7	102.8	104.1
Intraassay % CV for Bio-Rad controls				
Low	7.8	10.6	3.0	13.2
Medium	5.3	7.7	10.5	[1]
High	9.9	3.5	4.9	[1]
Interassay % CV for Bio-Rad controls				
Low	9.2	11.6	5.9	14.5
Medium	8.6	7.2	8	[1]
High	5.9	7.8	7.8	[1]
Linearity[2]				
(1:1)	26.1 IU/L	15.4 IU/L	28.4 ng/mL	61.5nmol/L
(1:2)	13.7 IU/L(105)	7.7 IU/L(100)	15.2 ng/mL(107)	31.2nmol/L(101)
(1:4)	7.4 IU/L(113)	3.95IU/L(103)	7.5 ng/mL(105)	12.6nmol/L (82)

Radioimmunoassays	T	E2	DHEA-S	A	C
Sensitivity	6.3 ng/dL	9 pg/mL	8.0 ng/mL	0.012 ng/mL	0.46 µg/dL
Average % recovery	100.7	103.4	98.6	105.5	94.6
Intraassay % CV for Bio-Rad controls					
Low	7.6	14.4	8.6	10.3	11.9
Medium	8.3	7.4	6.5	7.9	5.7
High	7	4	7.5	10.3	9.3
Interassay % CV for Bio-Rad controls					
Low	13.9	15	11.6	11.1	12.9
Medium	12.3	11.6	5.9	11.3	8.5
High	11.8	8.4	10.6	9.8	6.2
Linearity[2]					
(1:1)	292 ng/dL	170.9 pg/mL	457 ng/mL	0.91 mg/mL	21.6 µg/dL
(1:2)	130 ng/dL (89)	87.9 pg/mL (103)	229 ng/mL (100)	0.49 ng/mL (108)	11.6 ng/mL (107)
(1:4)	72 ng/dL (99)	42.0 pg/mL (98)	104 ng/mL (91)	0.21 ng/mL (92)	5.8 ng/mL (107)

[1] Concentrations same as low Bio-Rad.
[2] Numbers in parentheses are [(observed value × serial dilution factor)/expected value] × 100.

Precision. Precision was evaluated using blood spot controls and calculating intraassay coefficients of variation (CV) from concentrations of multiple samples assayed in a single assay and interassay CVs from concentrations determined for blood spot controls from multiple assays. Results for each assay are given in Table 5.

Linearity. Linearity was evaluated by serially diluting a high plasma sample with a sample of stripped human plasma. Aliquots of the high sample and dilutions were mixed 1:2 with washed red cells. A 50 µl aliquot was dropped onto the S&S paper, allowed to dry at room temperature overnight, and then stored in an airtight container at −20°C until assayed. For each assay, observed values and percentages of expected values for each dilution are reported in Table 5.

Accuracy, recovery. Varying concentrations of hormone were added to plasma samples originally containing lower and higher levels of endogenous hormone. Aliquots were mixed 1:2 with washed red cells and applied to the S&S paper as previously described. Average recoveries, calculated as percent of expected values, are listed in Table 5 for each assay.

Stability. For all assays, standards and controls that have been stored frozen at −20°C for at least one full year fall within a 10% CV range of the values determined immediately after preparation. Table 1 shows the length of time that samples remain within a 10% CV range of their initial value after having been stored for 1 week intervals at 4°C, room temperature, and 37°C for a

period of up to 8 weeks prior to freezing at −20°C.

Comparability to plasma samples. Whole bloods from venipunctures were spotted onto S&S papers, while portions were centrifuged and the plasmas (EDTA-anticoagulated) or serum (for the SHBG assay) withdrawn. Samples were evaluated for direct plasma/serum and blood spot measures. Table 4 shows Pearson correlation coefficients, r-square values, and the regression coefficients derived from simple linear regression analyses used to calculate plasma/serum equivalents from blood spot hormone concentrations.

MEASURING HORMONES FROM BLOOD SPOTS COLLECTED IN REMOTE FIELD SETTINGS: A FEASIBILITY STUDY AMONG THE HAGAHAI OF PAPUA NEW GUINEA

The Hagahai are a recently contacted (1984) forager-swiddenist population (n ~ 295) living on the northwest edge of the Schrader Range in Papua New Guinea (Jenkins et al., 1989). The lateness of contact reflects the remoteness of the site, at an altitude of 350–2400 m and accessible only by helicopter or by 1 week's walk from the nearest government post. In conjunction with regular health exams, demographic surveys, and other ongoing biomedical and ethnographic research headed by Carol Jenkins with the Papua New Guinea Institute for Medical Research (IMR), endocrine measures (from plasmas collected by venipunctures) and growth data (heights, weights, and skin folds) have been collected over a 10 year period to assess health status and acculturative and ecological impact on juvenile growth and development. Given the remoteness of the area, the logistical difficulties of sample collection and transport, and the technical field support of the IMR, the Hagahai site was an ideal location to test the feasibility of blood spot hormonal measures. Because the ongoing health surveillance protocol involved venipuncture, matched plasma and blood spot samples could be collected for comparative endocrine analysis. We had previously established that there was no difference in gonadotropin values assayed from blood spots produced by dropping whole blood from a finger prick vs. those produced with whole blood from a venipuncture (paired *t*-test of difference between finger-prick and venipuncture blood spot hormonal measures: *P* .17 for FSH, *P* .25 for LH). Immediate application of blood drops from the venipuncture onto the S&S papers prior to centrifugation of the sample therefore allowed production of matched plasma (EDTA-anticoagulated) and blood spot samples without any extra blood sampling.

On the day prior to departure for the field (accessed in this case by a 1 hour helicopter flight from Mt. Hagen), blood spot samples from the two authors and blood spot controls from commercial control products were prepared as described above at the laboratories of the IMR at Goroka, PNG. One set of controls and samples was dried overnight and then stored at −20°C. The other set of blood spots was taken to the field site and exposed to the same environmental conditions as the Hagahai samples that were collected over the next 8 days.

Field collection of samples proceeded as follows. Immediately following venipuncture, blood from the syringe was dropped onto the prelabeled S&S papers, and the remaining sample was placed in an EDTA collection tube. After centrifugation (with a generator-powered centrifuge), plasma was transferred to cryogenic vials and frozen immediately in a liquid nitrogen cryogenic freezer. Blood spot samples, on the other hand, were allowed to air-dry (approximately 4 h) and then placed in zip-lock bags containing dessicant and stored for an 8 day period at environmental temperatures (ranging from 22–28°C). At the end of the 8 day sampling period, all Hagahai and control samples were taken to the IMR and stored at −20°C. Approximately 2 months after collection, plasma and blood spot samples and controls were shipped frozen to the Laboratory for Comparative Human Biology, Emory University, where they were stored frozen at −20°C until assay. Pituitary, adrenal, and gonadal hormones (LH, A, and T) were measured in both blood spots and plasmas from 27 males and 14 females, 9–25 years of age. Excluded from statistical analysis are LH and T for nine boys whose

TABLE 6. Manufacturer's expected ranges (MER) for blood spot commercial controls compared to blood spot hormone values for samples stored frozen at the Institute for Medical Research, Goroka, Papua New Guinea (IMR) and to samples exposed to field conditions at the Hagahai site

Bio-Rad commercial controls	A (ng/mL)			LH (IU/L)			T (ng/dL)		
	MER	IMR	Field	MER	IMR	Field	MER	IMR	Field
Level I	0.10–0.44	0.32	0.35	1.3–2.2	1.7	1.9	33.2–58.8	47.4	50.2
Level II	1.11–1.72	1.36	1.55	7.5–11.2	8.3	8.4	499–645	583	501
Level III	3.70–5.89	3.87	4.19	21.2–31.5	26	24.1	[1]	[1]	[1]

[1] Concentrations exceed range of standard curve.

TABLE 7. Comparison of Hagahai plasma and plasma-equivalent blood spot results: Means and standard errors (SE) for plasma (Pl) and plasma-equivalent blood spot (P-E BS) values, Pearson correlation coefficients (r), r-squares (r^2), and P *values from simple linear regressions, and paired* t*-test* P *values*

	Mean (SE)	r	r^2	*P*	*t*-test
A (ng/mL)					
Pl	0.60 (0.41)				
P-E BS	0.65 (0.36)	0.90	0.81	<0.01	0.11
LH (IU/L)					
Pl	4.55 (11.8)				
P-E BS	4.82 (13.7)	0.99	0.98	<0.01	0.48
T (ng/dL)					
Pl	194.5 (146.7)				
P-E BS	214.8 (167.8)	0.98	0.95	<0.01	0.05

values fell below the sensitivity limit for the blood spot and plasma assays and results from two pregnant females (LH levels exceeding the standard curve range of 125 IU/L).

Table 6 shows expected ranges for blood spot controls and hormone measures from controls stored frozen at the IMR from time of preparation and those that were taken to the field and exposed to environmental conditions. Means and standard errors (SE) for both plasma and plasma-equivalent blood spot values, Pearson correlation coefficients (r), r-squares, and *P* values from simple linear regressions as well as paired *t*-test *P* values evaluating differences in plasma and plasma-equivalent blood spot values are shown in Table 7.

Assay results for all blood spot commercial controls—those kept frozen at the IMR and those that traveled to the field site—fell within the expected ranges. Similarly, hormone values for the sample aliquots stored at the IMR differed little from results from those taken to the field and treated identically to the samples collected at the field site. Further, strong correlations of matched plasma and blood spot samples, as shown in Table 7, suggest that blood spot samples represented as adequate a sampling method as the more cumbersome, costly, time-consuming, and invasive venipuncture plasma samples. Such results support our contention that blood spot sampling is a useful as well as practical field method for studying central and peripheral endocrine regulators of reproductive maturation and function.

RESEARCH APPLICATIONS OF BLOOD SPOT METHODS

In this section, we briefly describe a set of studies which illustrate the practical and scientific value of blood spot sampling. The logistical advantages discussed above allow a broader scope of endocrine research including more cross-cultural and population-based epidemiological studies on a variety of topics and research questions. Our experience has shown that large numbers of samples can be collected over short periods of time even in remote settings, that large longitudinal off-site epidemiological studies (even child studies) can be undertaken, that repeat samplings of both children and adults are possible, and that well-motivated and -instructed subjects can self-sample and mail sample papers directly to the lab for storage and analysis.

Cross-sectional developmental study

Blood spots offer advantages for developmental studies because they allow minimally invasive measurement, acceptable to child and parent, of the very low levels of regulatory protein and peripheral steroid hormones present through early puberty. Assay of these hormones is required for detecting and tracking puberty and provides

a basis for cross-sex and cross-population comparison of pubertal timing and progression. Accordingly, one of our first studies employing blood spots concerned characterization of endocrine and morphologic status over the juvenile period. The study, undertaken in collaboration with Nicholas Blurton Jones and with field collections by Jennifer Phillips-Davids (Phillips et al., 1991), involved the Hadza, a hunter-gatherer population of around 600 living in northwest Tanzania and subject of intensive ongoing investigation by Nicholas Blurton Jones, Kristin Hawkes, and colleagues (Blurton Jones et al., 1989; Blurton Jones, 1993). The Hadza are of interest in part because, unlike the intensively care-giving !Kung, they pursue a parental care strategy predicated on early child foraging and substantial self-provisioning by the end of the first decade (Blurton Jones, 1993). Like the !Kung, sex preference in child treatment is reported as minimal. Further, as government policy increasingly constrains Hadza to abandon foraging for farming, collection of baseline presedentization data was essential to provide a comparative basis for future studies of effects of changes in subsistence and lifestyle. Ease of collection, storage, and transport of blood spots allows such rapid cross-sectional surveys.

In the last 2 weeks of the 1991 summer field session, blood spot samples were collected from all available individuals ages 5–20 (n = 156). Samples were stored at ambient temperatures but away from heat sources for up to 24 days before return to our laboratory for storage at −23°C until analysis. Unsurprising in the light of reports on similar groups in the region was our finding that the Hadza are later maturing, showing a median age at significant elevation of LH (and inferentially of pubertal onset) of around 11.5 years in girls. But our finding of a dramatic delay of pubertal onset in boys (median age at significant LH elevation about 13.5 years), at least 2 years after girls (Fig. 1), was surprising and represents the largest sex difference in pubertal onset that we have as yet observed. For instance, we and others find that, among American youth, boys reach endocrine and morphologic puberty about 6 months after girls (reviewed in

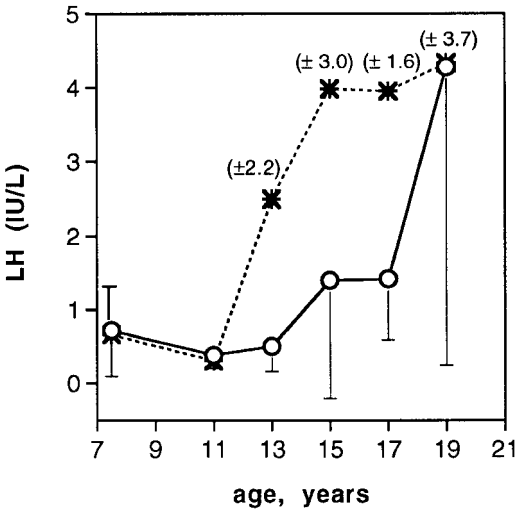

Fig. 1. Plasma equivalent blood spot LH in Hadza females (--*--) and males (—○—). Points represent age-grouped means (±SD, n = 5–13).

Angold and Worthman, 1993). Reports on secular trends to accelerated reproductive maturation are based largely on age at menarche (Eveleth and Tanner, 1990). Blood spot sampling offers a feasible method to track the developmental process and variation in both sexes and to probe the social, cultural, and ecological factors and changes that underlie such variation.

Longitudinal, population-based epidemiologic study

Use of blood spot sampling has allowed us to undertake the first large-scale population-based endocrine study of the relationship of normal puberty to emergence of sex differences and adult patterns of psychopathology and of the effect of environmental quality on normal variation in pubertal development in Western adolescents (Angold and Worthman, 1993; Worthman, 1995). The main study was initiated by Jane Costello for an investigation of mental health service needs for rural youth (Costello et al., 1996) and comprises a probability-based sample of 1,100 children, ages 9, 11, and 13 years, drawn from 11 counties of western North Carolina. Heights, self-rated Tanner scores, and blood spot hormonal measures of LH, FSH, T, E2, DHEA-S, and A are used to evaluate development. Children are inter-

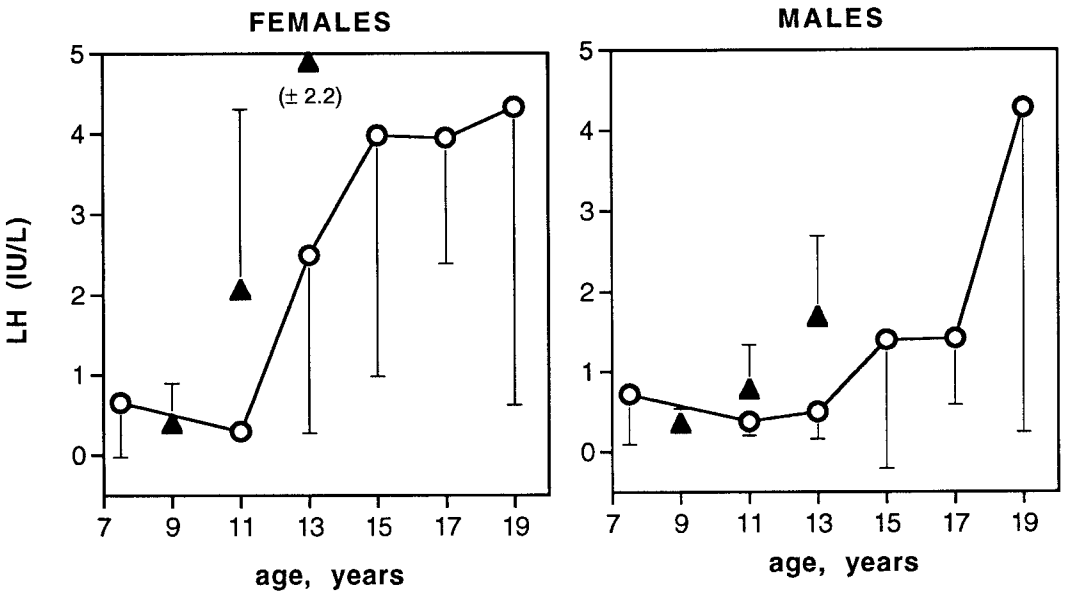

Fig. 2. Plasma equivalent blood spot LH for Hadza youth (---○---) compared to a large sample in western North Carolina (GSMS, ▲). Note that the points for Hadza represent age-grouped means (±SD, n = 5–13) by sex from cross-sectional data and have been connected to aid readability. Points for Americans represent ungrouped age-specific means (±SD) from a single year (year 1) of data collection.

viewed, sampled, and measured annually at home in conjunction with parallel parental interviews; blood spot sampling offered the only feasible method for hormone sampling by field teams, by offering ease of collection and transport, minimal instrumentation, and high child and parent acceptability. These features allowed incorporation of endocrine measures in an interview-based protocol concerned largely with ascertaining psychopathologic symptomatology, family functioning and socioeconomics, and mental health service use. For each annual hormonal profile, two finger pricks are performed 20 min apart to be averaged in later analysis for optimal representation of pulsatile hormones (Bain et al., 1988; Goldzieher et al., 1976) or analyzed separately for gauging dynamic responses. Samples are dried at room temperature and stored at 4°C for up to 2 weeks and then posted in express weekly shipments to the laboratory for storage at −23°C and subsequent assay.

We are currently in the third year of this ongoing study, but our blood spot sample analyses have so far provided the basis for characterizing the relationship of puberty to emerging sex difference in rates of depression (Angold et al., 1995), for probing the considerable degree of individual physiologic variation and its correlates (McDade et al., 1995; Stallings et al., 1995), and even for examining cortisol reactivity to novelty and physical stress in relation to immune function and rearing conditions (McDade et al., 1997). These epidemiologic data from a well-fed, healthy American population of adolescents also provide a substantial empirical basis for population comparisons. By illustration, in Figure 2, the endocrine profile of Hadza youth are overlaid with means and ranges for our North Carolina sample. Comparison of blood spot plasma equivalent values, in this case for LH, shows that Hadza of both sexes enter puberty well after American children. Increases in mean LH occur later among Hadza, by about 2 years in girls and nearly 3.5 years in boys; the contrast of Hadza and American boys underscores the markedly delayed pubertal onset of the former. We do not have a ready explanation for pronounced male developmental delay among Hadza, though aspects of behavior, social ecology, or sex-differenti-

ated sensitivity to specific conditions may play a role. Nonetheless, without the endocrine data, we would not have distinguished this phenomenon so readily, nor would we have been able to distinguish whether it was centrally mediated (based on LH levels) or due to gonadal refractoriness (based on gonadal output); ability to monitor both pituitary regulatory hormones (LH in this case) as well as those of peripheral target organs (such as T or E2) allows discrimination across levels of endocrine regulation. Blood spot sampling provides a window to the set points and dynamics of brain regulation of reproductive maturation and adult function.

Dynamic study: Serial measures of endocrine response

Reproductive ecology is concerned with behavior, both in terms of its direct impact on reproductive function (Cumming et al., 1994) and vice versa (Campbell and Leslie, 1995). Despite the centrality of behavior as a bridge between culture or human ecology and reproductive performance or fertility, current reproductive ecology lacks endocrine studies of behavior-biology interactions. Blood spot measures facilitate such studies because they allow serial sampling to monitor central and peripheral dynamics both acutely and through time.

For reproductive ecology, a classic instance of biobehavioral dynamics is the suppressive effect of breast-feeding on ovarian function, a centrally mediated effect reflected in patterns of suckling-induced PRL release. In collaboration with Catherine Panter-Brick, we recently used blood spot sampling to examine the postsuckling trajectory of circulating PRL in two sympatric Nepali groups, the Tamang and Kami. Mothers in these two groups exhibit similar breast-feeding frequency and timing of supplementation but have quite different interbirth intervals: Tamang women, despite their greater height and weight, have average birth intervals 8 months longer than Kami (29 months) (Panter-Brick, 1991). The disparity may be due in part to heavy workloads and cyclic weight loss among Tamang women (Panter-Brick et al., 1993), but the group difference in duration of birth spacing (Panter-Brick, 1991) suggested a role for differences in endocrine effects of nursing.

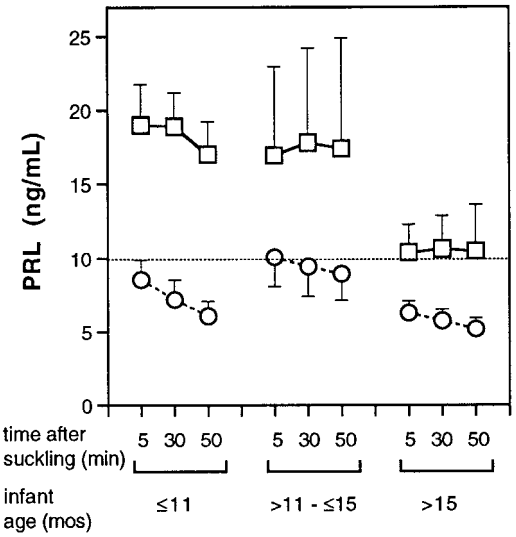

Fig. 3. Circulating prolactin concentrations (mean ± s.e.) at specified intervals after suckling (5, 30, and 50 min) by infant age (≤11, 11–≤15, and >15 months) in two sympatric Nepali groups, the Tamang (---□---) and the Kami (—○—).

In August 1991, we obtained serial fingerprick blood spot samples from all available nursing mothers (55 Tamang and 17 Kami) at 5, 30, and 50 min after termination of a nursing bout. Samples were air-dried and stored with drierite at ambient temperature until shipment to the laboratory, where they were stored at −23°C within 3 weeks after collection. Predictably, the magnitude and duration of suckling-induced PRL increase decreased with time postpartum, but groups differed in the rate of both declines (Fig. 3). Pituitary responsiveness to suckling decayed more rapidly among Kami both with time after nursing and by infant age. Underlying group differences in neuroendocrine function are contraindicated by the absence of group difference in immediate postsuckling (5 min) prolactin levels among mothers with young infants (≤11 mos.), though Kami showed significantly lower PRL by 50 min postsuckling in mothers with infants less than 22 months old. Further analyses suggested that PRL levels relate to different variables for the two groups, extrinsic (nurs-

ing bout length, infant age) for Tamang and intrinsic (maternal age) for Kami (Stallings et al., in press). We found that postsuckling PRL levels were highly predictive of maintenance of postpartum amenorrhea: Tamang and Kami women who maintained elevated PRL levels (≥10 ng/ml) as long as 50 min postsuckling were five times more likely to be amenorrheic (Stallings et al., 1996).

Serial hormone measures offer considerable potential for characterizing the role of behavior in reproductive ecology in men (Worthman and Konner, 1987) as well as women. We have recently completed collections for a study with Virginia Vitzthum of acute endocrine responses to exercise by menstrual cycle phase among Bolivian women at high altitude. This experience and that with Cynthia Beall in collections also undertaken in Bolivia (Worthman et al., 1997) suggest that finger-prick sampling is acceptable even to people in Andean regions long known for aversion to venipuncture.

Comparative life span endocrinology

Comparative endocrine analyses inform growing interest in the underlying social, cultural, and ecological factors that influence variation in the ontogeny of reproductive function. Recent studies of nonpregnant, nonlactating women across populations have revealed significant variation in average levels of ovarian hormones (Ellison, 1994), and intrapopulation studies have linked ovarian hormone variation to differences in energy balance (weight, workload) (Ellison, 1994; Ellison et al., 1989; Jasienska and Ellison, 1993; Panter-Brick et al., 1993). Comparative studies have shown that differences in the timing and course of pubertal development can be linked to variation in environmental quality and that such ecological variation can be quite marked across populations (Worthman, 1995), within populations (Riley, 1994; Laska-Mierzejewska, 1995; Stallings et al., 1995; Wood, 1994), and even within families or households (Worthman, 1996, in press).

Cumulatively, these studies suggest the value to reproductive ecology of a life history, life span approach which incorporates reproductive ontogeny with adult function and aging. Such a perspective addresses three related issues in relation to their impact on central neuroendocrine regulation and gonadal function: 1) how ecological and behavioral factors affect the onset and trajectory of reproductive development, 2) how divergent developmental histories condition the effect of ecological and behavioral factors on adult reproductive function, and 3) how these histories shape processes of senescence. This contextual ontogenetic approach should help determine whether divergent life histories condition the effect of proximal factors on reproductive processes that generate variation in fertility.

Blood spot sampling, for both its convenience and range of readable hormones, enhances the ability to study groups from widely variant environments and reproductive life histories and thus promotes comparative endocrinological research. The potential of this approach can be exemplified by a comparison of two populations of Papua New Guinea, the Hagahai and the Amele, studied in collaboration with Carol Jenkins of the IMR (Jenkins et al., 1989; Worthman et al., 1993). The Hagahai, described briefly above, are a demographically declining population plagued by steep morbidity and mortality rates and poor nutrition, particularly among women and children; these conditions are reflected in stunted growth, late maturation, and small adult body size. The Amele, by contrast, are a numerous subcoastal people who enjoy relatively good health care and nutrition, lower mortality, and concomitantly earlier maturation. Through ongoing work by Jenkins and the IMR, we had assembled a large series of plasma samples from Hagahai, and we used blood spot sampling to rapidly obtain a cross-sectional age-stratified sample set for Amele. Sample collection was initiated by Jim Rilling of our laboratory and the survey collected by Daina Lai of the IMR in August 1994. Such cross-sectional survey approaches are useful for studying juveniles and men, but the variability of endocrine profile in relation to women's reproductive state problematizes a survey approach to characterization of reproductive endocrinology of women in the reproductive years. Reproductive hormone values for young Hagahai (n = 21) and Amele (n = 36) men ages 21–25 years

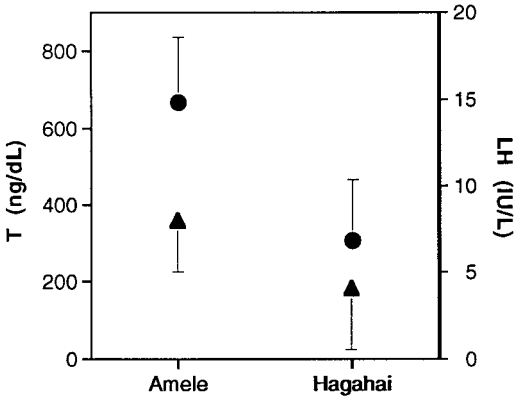

Fig. 4. Group variation in hypothalamo-pituitary-gonadal set points, showing differences between two Papua New Guinea groups, Hagahai and Amele, in T (---●---) and LH (—▲—) concentrations (mean ± SD) in young men ages 21–25 years.

are plotted in Figure 4. Selection of this age range avoids the issue of population differences in maturational schedule and allows comparison of the hypothalamo-pituitary-gonadal (HPG) axis at the point of peak young adult function. The population difference is readily apparent: young Amele men have twice the testosterone and the LH of their Hagahai counterparts. That the population difference in T and LH is symmetrical is significant, for it indicates that the disparity in testicular output is centrally mediated, not a product of gonadal refractoriness or impairment. In other words, the HPG axis is downregulated among Hagahai relative to Amele men.

These observations furthermore demonstrate population differences in gonadal output of steroids known to be linked not only to behavior and cognition but also to long-term health outcomes, including risk for reproductive cancers. Hagahai men clearly will have less quantitative lifetime exposure to testosterone and its metabolites than will Amele, not only because they mature later than do Amele but also because testicular output is regulated to a lower level. Similar effects may obtain in women. Dramatically escalating rates of reproductive cancers, particularly breast cancer (Kelsey and Horn-Ross, 1993), have spurred concern with the social-ecological transformations that may impair women's reproductive function (Lasley et al., 1994) and heighten risk for neoplasia (Eaton et al., 1994). Population variation in breast cancer rates has been related to differences in serum levels of ovarian hormones (Pike et al., 1993; Bernstein and Ross, 1993). Comparisons of premenopausal Western women and Asian women have overwhelmingly found higher estrogen levels in Western women (Bernstein and Ross, 1993). Recent anthropological studies of Lese women of Zaire, the Tamang of Nepal, and rural farm women in southern Poland have found lower average mid-luteal progesterone levels than among Boston women of similar ages (Ellison, 1994). Differences in diet or energy expenditure may account for some of these divergent endocrine patterns (Ellison, 1994; Bernstein and Ross, 1993).

Recognition of population differences and within-population secular trends in gonadal steroid exposure has raised keen interest in the magnitude, causes, and consequences of such variation. The blood spot SHBG method reported here should also assist such investigation, as levels of this binding protein strongly influence bioavailable circulating steroid concentrations. Indeed, variation in SHBG values has been linked to breast cancer risk. Lower levels of SHBG have been found in high-risk groups, such as obese women (Anderson, 1974; Bernstein and Ross, 1993; Moore et al., 1987), women with early age at menarche and established regularity of menstrual cycles (Apter and Vihko, 1989; Bernstein and Ross, 1993; Siiteri and Simberg, 1986), and nulliparous vs. parous premenopausal women (Bernstein et al., 1985; Moore et al., 1987). Although some associations have been found between breast cancer and progesterone, PRL, and the androgens T and DHEA-S, studies of these hormones are less extensive and findings less consistent (Bernstein and Ross, 1993).

Beyond reproductive ecology

Applications of the blood spot sampling methods described here, available elsewhere, or currently under development in our laboratory contribute to biosocial and behavioral research outside reproductive ecology as well. To briefly indicate the scope of such applications, we highlight four areas of investigation in stress, immune function,

metabolism, and multiple or cross-axis endocrine function. First, concerning stress, the blood spot cortisol measure reported here can be deployed for population comparisons or dynamic studies of individuals. For instance, the 20 min dual blood spot sampling protocol used in our North Carolina study, described above, also allows characterization of adrenocortical responses to experience. Because we sample at 20–30 min into the home interview and a cortisol response takes about 15–20 min to peak in circulation, our sample at time one provides a measure of response to social novelty (viz., unknown interviewers coming into the home), while that at time two indexes the response to the stress of the finger prick at time one. We have found strong sex differences and developmental effects in degree of cortisol responsiveness to social novelty (McDade et al., 1997). Thus, careful sampling design that takes advantage of the interventions introduced by the research protocols themselves can provide a window to the dynamics of stress responsiveness.

Although saliva sampling offers real advantages for stress research and is commonly used in this area (Pollard, 1995), blood spot sampling allows a wider array of physiologic end points to be probed in tandem. For example, large proteins such as antibodies are also stable and measurable in blood spots, which opens a window to immune function, a central actor in adaptation to pathogens and thus a core determinant of well-being and disease vulnerability. Measures of cortisol in the North Carolina study were coupled with those of antibody to resident Epstein Barr virus that varies in inverse proportion with immune competence to reveal an effect of environmental quality on immunocompetence (McDade et al., 1997). Psychosocial and material adversity was found to depress immunocompetence, but both strong sex differences and developmental effects on this relationship were also identified. Another published use of protein measures in blood spots for anthropological purposes is for ABO blood group typing (Aebischer et al., 1990).

Third, more could be done with the well-established measures of thyroid function (TSH, T3, T4) to track metabolic regulation as a regulator of energy consumption, a marker of nutritional deficiency, and a factor in aging. As a simple instance of this application, our measure of blood spot TSH for Cynthia Beall's study of adaptation to high seasonal variation in food intake and thermal loads in Tibetans allowed us to establish that hypothyroidism could not explain the lack of seasonal variation in basal metabolic rates in this population (Beall et al., 1996).

Lastly, the wide range of hormones measurable in blood spots facilitates expansion of research on endocrine regulation, adaptation, and response to comparative analysis across endocrine axes. Reproductive ecology has scarcely posed the question of whether or how effects of nutritional constraints on gonadal function may be moderated or even mediated by the ability to make metabolic adjustments. Nor have we considered whether individual, intrapopulation variation in reproductive hormone function or reproductive output may correlate with the adrenal androgen markers (DHEA, DHEAS) linked to longevity. Outside of reproductive ecology, the literature on stress documents a relationship between temperamental differences in cortisol responsiveness to differences in neuroendocrine functioning (serotoninergic and noradrenergic) that in turn relate to differential morbidity and mortality risks that are both behaviorally and physiologically mediated (Barr et al., 1994; Williams, 1994). Comparative studies of these dynamics, and of the effects of varying social and ecological environments on neuroendocrine function, temperament, and resulting differential well-being may be more feasible with blood spot sampling. A primary function of endocrine systems is to set physiologic priorities and allocate limited resources within the body to meet current functional demands and constraints; the common occurrance of behavioral, psychosocial, or other environmental challenges means that demands of all physiologic systems can rarely be met simultaneously but must be juggled, traded off, and optimized over the short, medium, and long term. Investigation of genetic and facultative or developmentally induced variation in relationships among regulatory axes with respect to differential performance or well

being is still in its infancy (but see Finch and Rose, 1995) but bids fair to yield a more complete explanatory framework for endocrine function.

CONCLUSIONS

The ability to measure hormones across populations living in diverse physical and social environments presents an opportunity to probe the extent, causes, and consequences of human variation in endocrine physiology and to expand our understanding of "normal" human endocrine function in everyday life. The present report documents methodological details, sample stabilities, assay performance, and other technical aspects of the sampling and assay methods. We have illustrated the research advantages of finger-prick sampling and blood spot assays for diverse study designs, including rapid cross-sectional surveys, large epidemiologic and longitudinal studies, serial sampling, and self-sampling by subjects. The availability of highly sensitive, accurate, and precise assays for determination of a wide range of hormones (gonadotropins, gonadal and adrenal steroids, and SHBG) from a very small blood sample (200 µl of whole blood) provides the opportunity for minimally invasive, maximally convenient ascertainment of developmental or reproductive status and an index of bioavailable gonadal steroids.

Population variation represents an important source of information about biological mechanisms, about responsiveness of the endocrine system to environmental influences, about ontogenetic and acute sources of variation, and about the consequences of this variation for human function and well-being. Furthermore, blood spot sampling, especially self-collections, provides an opportunity to integrate hormonal studies into a wide range of designs, from small samples from remote or low-tech anthropological field settings to public health surveillance programs that provide large data bases on demographics, nutrition, socioeconomics, and reproductive histories. Thus, availability of practical collection methods and improved hormonal assays permit identification of environmental, social, occupational, and behavioral effects on reproductive processes and reproductive health in particular, while they also expand the potential scope of endocrine research in general.

ACKNOWLEDGMENTS

We gratefully acknowledge the collaboration of several investigators central to the field studies described here. They are Adrian Angold, Cynthia Beall, Nicholas Blurton Jones, Ben Campbell, Jane Costello, David Gubernick, Carol Jenkins, and Catherine Panter-Brick. We also thank Patricia Castro, Daina Lai, Jennifer Phillips Davids, and Jim Rilling for excellent technical or field assistance. Finally, we recognize the essential contributions of the people who participated in the studies: the Amele, Hagahai, Hadza, Tamang and Kami, and Americans of western North Carolina and of central Wisconsin. This work was partially supported by a W.T. Grant Foundation faculty scholarship to C.M.W.; W.T. Grant Foundation grants 92-1489-92 and 94-1489-2; University Research Committee of Emory University; and NIMH MH48085 (to Costello). Reagents for initial development of the PRL, FSH, and LH assays were donated by Wallac, Inc.

LITERATURE CITED

Aebischer ML, Martorana MC, Costa F, Battaggia C, Madera A, Destito D, Machera F, Bailly C, and Angeloni P (1990) Evaluation of the sensitivity of microfilter paper assays in an anthropological study: Results of samples from Cameroon and Tanzania. Anthropol. Anz. *48:*15–23.

Anderson D (1974) Sex-hormone-binding-globulin. Clin. Endocrinol. *3:*69–96.

Angold A, and Worthman CM (1993) Puberty onset of gender differences in rates of depression: A developmental, epidemiologic and neuroendocrine perspective. J. Affect. Disord. *29:*145–158.

Angold A, Costello EJ, and Worthman CM (1995) Sex-differentiated effects of puberty on the relationship between adversity and symptoms of depression: A report from the Great Smoky Mountains Study. Am. J. Phys. Anthropol. *20*(Suppl.):58 (abstract).

Apter D, and Vihko RJ (1989) Some endocrine characteristics of early menarch, a risk factor for breast cancer, are preserved into adulthood. Int. J. Cancer *44:* 783–787.

Bain J, Sanders RM, Langevin R, Hucker S, and D'Costa M (1988) Serum pituitary and steroid hormone levels in the adult male: One value is as good as the mean of three. Fertil. Steril. *49:*123–126.

Barr RG, Boyce WT, and Zeltzer LK (1994) The stress-illness association in children: A perspective from the

biobehavioral interface. In RJ Haggerty, LR Sherrod, M Garmezy, and M Rutter (eds.): Stress, Risk, and Resilience in Children and Adolescents. Cambridge: Cambridge University Press, pp. 182–224.

Bassett F, Gross B, and Eastman C (1986) Radioimmunoassay of prolactin in blood spotted on filter paper. Clin. Chem. *32:*854–856.

Beall C, Worthman CM, Stallings J, Strohl K, Brittenham G, and Barragan M (1992) Salivary testosterone concentration of Aymara men native to 3600 m. Ann. Hum. Biol. *19:*67–78.

Beall CM, Henry J, Worthman CM, and Goldstein MC (1996) Basal metabolic rate and dietary seasonality among Tibetan nomads. Am. J. Hum. Biol. *8:*361–370.

Bernstein L, and Ross R (1993) Endogenous hormones and breast cancer risk. Epidemiol. Rev. *15:*48–65.

Bernstein L, Pike MC, Ross RK, Judd HL, Brown JB, and Henderson BE (1985) Estrogen and sex hormone–binding globulin levels in nulliparous and parous women. J. Natl. Cancer Inst. *74:*741–745.

Blank B, Attanasio A, Rager K, and Gupta D (1978) Determination of serum sex hormone binding globulin (SHBG) in preadolescent and adolescent boys. J. Steroid Biochem. *9:*121–125.

Blurton Jones N (1993) The lives of hunter-gatherer children: Effects of parental behavior and parental reproductive strategy. In ME Pereira and LA Fairbanks (eds.): Juvenile Primates. New York: Oxford University Press, pp. 309–326.

Blurton Jones N, Hawkes K, and O'Connell JF (1989) Measuring and modelling costs of children in two foraging societies: Implications for schedule of reproduction. In V Standen and R Foley (eds.): Comparative Socioecology of Mammals and Humans. Oxford: Blackwell, pp. 367–390.

Brombacher PJ, Henkel E, Dessler AC, and Van Dieijen-Visser MP (1988) Multicentre study of a new test for TSH-screening in blood spots. Ann. Clin. Biochem. *25:*530–535.

Campbell BC, and Leslie PW (1995) Reproductive ecology of human males. Yearb. Phys. Anthropol. *21:*1–26.

Campbell KL (1994) Blood, urine, saliva, and dip-sticks: Experiences in Africa, New Guinea, and Boston. Ann. N. Y. Acad. Sci. *709:*312–330.

Carter G, Holland S, Alaghband-Zadeh J, Rayman G, Dorrington-Ward P, and Wise P (1983) Investigation of hirsutism: Testosterone is not enough. Ann. Clin. Biochem. *20:*262–263.

Costello EJ, Angold A, Burns B, Stangl DK, Tweed DL, Erkanli A, and Worthman CM (1996) The Great Smoky Study of Youth: Goals, design, methods, and the prevalence of DSM-III-R disorders. Arch. Gen. Psychiatry *53:*1129–1136.

Cumming D, Wheeler G, and Harber V (1994) Physical activity, nutrition, and reproduction. Ann. N. Y. Acad. Sci. *709*:55–76.

Dunkel L, Alfthan H, Stenman UH, Tapanainen P, and Perheentupa J (1990) Pulsatile secretion of LH and FSH in prepubertal and early pubertal boys revealed by ultrasensitive time-resolved immunofluorometric assays. Pediatr. Res. *27:*215–219.

Dunn J, Nisula B, and Rodbard D (1981) Transport of steroid hormones. Binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid-binding globulin in human plasma. J. Clin. Endocrinol. Metab. *53:*58–68.

Eaton S, Pike M, Short R, Lee N, Trussell J, Hatcher R, Wood J, Worthman C, Blurton Jones N, Konner M, Hill K, Bailey R, and Hurtado A (1994) Women's reproductive cancers in evolutionary context. Q. Rev. Biol. *69:*354–367.

Egan S, Betts P, Thomson S, Wallace AM, and Wood PJ (1989) A blood spsot androstenedione assay suitable for home monitoring of steroid replacement therapy in congenital adrenal hyperplasia. Ann. Clin. Biochem. *26:*262–267.

Ellison P (1988) Human salivary steroids: Methodological considerations and applications in physical anthropology. Yearb. Phys. Anthropol. *31:*115–142.

Ellison P (1994) Salivary steroids and natural variation in human ovarian function. Ann. N. Y. Acad. Sci. *709:*287–298.

Ellison P, Peacock N, and Lager C (1989) Ecology and ovarian function among Lese women of the Ituri Forest, Zaire. Am. J. Phys. Anthropol. *78:*519–526.

Eveleth PB, and Tanner JM (1990) Worldwide Variation in Human Growth, 2nd ed. Cambridge: Cambridge University Press.

Finch CE, and Rose MR (1995) Hormones and the physiological architecture of life history evolution. Q. Rev. Biol. *70:*1–52.

Goldzieher JW, Tazewell SD, Smith KD, and Steinberger E (1976) Improving the diagnostic reliability of rapidly fluctuating plasma hormone levels by optimized multiple-sampling techniques. J. Clin. Endocrinol. *43:*823–830.

Haavisto AM, Dunkel L, Pettersson K, and Huhtaniemi I (1990) LH measurements by in vitro bioassay and a highly sensitive immunofluorometric assay improve the distinction between boys with constitutional delay of puberty and hypogonadism. Pediatr. Res. *27:* 211–214.

Hofman LF, Klaniecki JE, and Smith EK (1985) Direct solid-phase radioimmunoassay for screening 17 alpha-hydroxyprogesterone in whole-blood samples from newborns. Clin. Chem. *31:*1127–1130.

Jasienska G, and Ellison P (1993) Heavy workload impairs ovarian function in Polish peasant women. Am. J. Phys. Anthropol. *16:*117–118 (abstract).

Jenkins C, Dimitrakakis M, Cook I, Sanders R, and Stallman N (1989) Culture change and epidemiological patterns among the Hagahai, Papua New Guinea. Hum. Ecol. *17:*27–57.

Kelsey J, and Horn-Ross P (1993) Breast cancer: Magnitude of the problem and descriptive epidemiology. Epidemiol. Rev. *15:*7–16.

Knudsen RC, Slazyk WE, Richmond JY, and Hannon WH (1993) Guidelines for the shipment of dried blood spot specimens. Infant Screening *16:*1–4.

Kraiem Z, Kahana L, Elias V, Ghersin S, and Sheinfeld M (1980) Radioimmunoassay of cortisol in blood collected on filter paper. Clin. Chem. *26:*1916–1917.

Laska-Mierzejewska T (1995) Age at menarche as an indicator of the socioeconomic situation of rural girls in Poland in 1967, 1977, and 1987. Am. J. Hum. Biol. *7:*651–656.

Lasley B, Mobed K, and Gold E (1994) The use of urinary hormonal assessments in human studies. Ann. N. Y. Acad. Sci. *709:*299–311.

Lovgren T, Hemmila I, Pettersson K, and Halonen P (1985) Time-resolved fluorometry in immunoassay. In WP Collins (ed.): Alternative Immunoassays. Chichester: John Wiley and Sons, pp. 203–217.

Malamud D, and Tabak L (eds.) (1993) Saliva as a Diagnostic Fluid. New York: New York Academy of Sciences.

Marshall J, Dalkin A, Haisenleder D, Paul S, Ortolano G, and Kelch R (1991) Gonadotropin-releasing hormone pulses: Regulators of gonadotropin synthesis and ovulatory cycles. Rec. Prog. Horm. Res. *47:*155–198.

McDade T, Worthman CM, Angold A, Costello EJ, and Stallings, J (1995) Physiologic bases of individual

variation in pubertal timing and progression: Report from the Great Smoky Mountains Study. Am. J. Phys. Anthropol. *20*(Suppl.):148 (abstract).

McDade TW, Stallings JF, and Worthman CW (1997) Psychosocial stress and cell-mediated immune function: validation of a blood spot method for Epstein-Barr virus antibodies. Am. J. Phys. Anthropol. Suppl. 24:164–5.

Meredith NK, and Hannon WH (1993) Preparation of dried blood spot materials for quality assurance of assays for antibodies to human immunodeficiency virus. In BL Therrell (ed.): Laboratory Methods for Neonatal Screening. Washington, DC: American Public Health Association, pp. 225–241.

Moore JW, Key TJA, Bulbrook RD, Clark GM, Allen DS, Wang DY, and Pike MC (1987) Sex hormone binding globulin and risk factors for breast cancer in a population of normal women who had never used exogenous sex hormones. Br. J. Cancer *56:*661–666.

National Committee for Clinical Laboratory Standards (NCCLS) (1992) NCCLS Approved Standard LA4-A2. Blood Collection on Filter Paper for Neonatal Screening Programs. Illanova, PA: National Committee for Laboratory Standards.

Osredkar J, Vrhovec I, Jesenovec N, Kocijancic A, and Prezelj J (1989) Salivary free testosterone in hirsutism. Ann. Clin. Biochem. *26:*522–526.

Panter-Brick C (1991) Lactation, birth spacing and maternal work-loads among two castes in rural Nepal. J. Biosoc. Sci. *23:*137–154.

Panter-Brick C, Lotstein DS, and Ellison PT (1993) Seasonality of reproductive function and weight loss among rural Nepali women. Hum. Reprod. *8:*684–690.

Pardridge W (1981) Transport of protein-bound hormones into tissues in vivo. Endocr. Rev. *2:*102–123.

Petra P (1991) The plasma sex steroid binding protein (SBP or SHBG). A critical review of recent developments on the structure, molecular biology and function. J. Steroid Biochem. Mol. Biol. *40:*735–753.

Petsos P, Ratcliffe WA, Heath DF, and Anderson DC (1986) Comparison of blood spot, salivary and serum progesterone assays in the normal menstrual cycle. Clin. Endocrinol. *24:*31–38.

Phillips J, Worthman CM, and Stallings JF (1991) New field techniques for detection of female reproductive status. Am. J. Phys. Anthropol. *85:*143 (abstract).

Pike M, Spicer D, Dahmoush L, and Press M (1993) Estrogens, progestogens, normal breast cell proliferation, and breast cancer risk. Epidemiol. Rev. *15:*17–35.

Pollard T (1995) Use of cortisol as a stress marker: Practical and theoretical problems. Am. J. Hum. Biol. *7:*265–274.

Riad-Fahmy D, Read G, Walker R, Walker S, and Griffiths K (1987) Determination of ovarian steroid hormone levels in saliva: An overview. J. Reprod. Med. *32:*254–272.

Riley A (1994) Determinants of adolescent fertility and its consequences for maternal health, with special reference to rural Bangladesh. Ann. N. Y. Acad. Sci. *709:*86–100.

Rilling JK, Worthman CM, Campbell BC, Stallings JF, and Mbizva M (1996) Ratios of plasma and salivary testosterone throughout puberty: production vs. bioavailability. Steroids *61:*374–378.

Ruutiainen K, Sannikka E, Sanntti R, Erkkola R, and Adlercreutz H (1987) Salivary testosterone in hirsutism: Correlations with serum testosterone and the degree of hair growth. J. Clin. Endocrinol. Metab. *65:*1015–1020.

Sannikka E, Terho P, Suominen J, and Santti R (1983) Testosterone concentrations in human seminal plasma and saliva and its correlation with non-protein bound and total testosterone levels in serum. Int. J. Androl. *6:*319–330.

Siiteri P, Murai J, Hammond G, Nisker J, Raymoure W, and Kuhn R (1982) The serum transport of steroid hormones. Rec. Prog. Horm. Res. *38:*457–503.

Siiteri PK, and Simberg NH (1986) Changing concepts of active androgens in blood. Clin. Endocrinol. Metab. *15:*247–258.

Stallings J, Angold A, Costello EJ, and Worthman CM (1995) Environmental effects on pubertal development in western adolescents: Report from the North Carolina Great Smoky Mountain Study. Am. J. Phys. Anthropol. *20*(Suppl.):201 (abstract).

Stallings JF, Worthman CM, Panter-Brick C, and Coates RJ (1996) Prolactin response to suckling and maintenance of postpartum amenorrhea among intensively breastfeeding Nepali women. Endocr. Res. *22:*1–28.

Stallings JF, Worthman CM, and Panter-Brick C (in press) Biological and behavioral factors influence caste differences in prolactin levels among breastfeeding Nepali women. Am. J. Hum. Biol.

Stenman UH, Alfthan H, Koskimies A, Seppala M, Pettersson K, and Lovgren T (1985) Monitoring the LH surge by ultrarapid and highly sensitive immunofluorometric assay. Ann. N. Y. Acad. Sci. *442:*554–550.

Thomson S, Wallace AM, and Cook BA (1989) ^{125}I-radioimmunoassay for measuring androstenedione in serum and in blood-spot samples from neonates. Clin. Chem. *35:*1706–1712.

Torresani TE, and Scherz T (1986) Neonatal thyroid screening by a non-radioactive method: Evaluation of thyrotropin time-resolved fluoroimmunoassay. Clin. Chem. *32:*1013–1016.

Vining R, and McGinley R (1987) The measurement of hormones in saliva: Possibilities and pitfalls. J. Steroid Biochem. *27:*81–94.

Waite KV, Maberly GF, and Eastman CJ (1987) Storage conditions and stability of thyrotropin and thyroid hormones on filter paper. Clin. Chem. *33:*853–855.

Wang C, Plymate S, Nieschlag E, and Alvin-Paulsen C (1981) Salivary testosterone in men: Further evidence of a direct correlation with serum testosterone. J. Clin. Endocrinol. Metab. *53:*1021–1024.

Williams RB (1994) Neurobiology, cellular and molecular biology, and psychosomatic medicine. Psychosom. Med. *56:*308–315.

Wood JW (1994) Dynamics of Human Reproduction: Biology, Biometry, Demography. Hawthorne, NY: Aldine de Gruyter.

Worthman CM (1995) Epidemiology of human development. Am. J. Hum. Biol. *7:*138–139 (abstract).

Worthman CM (1996) Biosocial determinants of sex ratios: survivorship, selection, and socialization in the early environment. In SJ Ulijaszek and CJK Henry (eds.): Long-Term Consequences of Early Environments. Cambridge: Cambridge University Press, pp. 45–68.

Worthman CM (in press) Evolutionary perspectives on the onset of puberty. In WR Trevathan, JJ McKenna, and EO Smith (eds.): Evolutionary Medicine. Oxford University Press.

Worthman CM, and Konner MJ (1987) Testosterone levels change with subsistence hunting effort in !Kung San men. Psychoneuroendocrinology *12:*449–458.

Worthman CM, and Stallings JF (1994) Measurement of gonadotropins in dried blood spots. Clin. Chem. *403:* 448–453.

Worthman CM, Stallings JF, and Gubernick D (1991)

Measurement of hormones in blood spots: A non-isotopic assay for prolactin. Am. J. Phys. Anthropol. *85:*186–187.

Worthman CM, Jenkins C, Stallings JF, and Lai D (1993) Atenuation of nursing-related ovarian suppression and high fertility in well-nourished, intensively breast-feeding Amele women of lowland Papua New Guinea. J. Biosoc. Sci. *25:*425–443.

Worthman CM, Beall CM, and Stallings JF (1997) Population variation in reproductive function of men. Am. J. Phys. Anthropol. *24(Suppl):*246.

Wu F, Butler G, Kelnar C, Stirling H, and Huhtaniemi I (1991) Patterns of pulsatile luteinizing hormone and follicle-stimulating hormone secretion in prepubertal (midchildhood) boys and girls and patients with idiopathic hypogonadotropic hypogonadism (Kallman's syndrome): A study using an ultrasensitive time-resolved immunofluorometric assay. J. Clin. Endocrinol. Metab. *72:*1229–1237.

Yalow R, and Berson S (1971) Principles of Competitive Protein Binding Assays. Philadelphia: JB Lippincott Co.